EXHIBIT B

If, in the absence of amiloride, all pores were permanently open, a Na current $I_{Na} = iN = 2000$ μa/cm^2 would result at 60 mM $(Na)_o$. This current is 75 times larger than the I_{Na} value of 26 μa/cm^2 actually observed (see legend to Fig. 1A). We deduce from this observation that even in the absence of amiloride the pores are not permanently open (*11*).

B. LINDEMANN

Second Department of Physiology, Universität des Saarlandes, 665 Homburg, West Germany

W. VAN DRIESSCHE

Laboratorium voor Fysiologie, 3000 Leuven, Belgium

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2. We suggest that (i) the term carrier be applied only to translocators where the transfer-mediating moiety moves through the lipid phase; (ii) the term pore be applied to water-filled pathways that permit diffusional transport, which can, however, involve binding of ions to parts of the pore structure; and (iii) different terms be used for translocators that do not meet these specifications [see also (*9*)].
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4. Under short-circuit conditions, the voltage across the apical membrane will come even closer to the transepithelial voltage when the resistance of the apical membrane is increased by amiloride. Also, the decrease of I_{Na} due to amiloride will decrease the K current of the K-selective, inward-facing membrane, and thus decrease the possible effect of this membrane on the recorded fluctuations. Finally, the intracellular Na concentration can be expected to become small in the presence of amiloride. This condition simplifies the computation of channel conductance.
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9. Theoretically, translocators that combine properties of carriers and pores are conceivable. For instance, at a transport site the membrane may effectively be thinned down to a narrow protein structure, part of which can bind an ion selectively and transfer it by a rotational movement in low-viscosity surroundings ["translocase"; see P. Mitchell, *Nature (London)* **180**, 134 (1957)]. Transfer rates may be high, although this is not very likely in view of the low turnover numbers of most enzymes. H. Passow (personal communication) has estimated turnover numbers of 2×10^4 sec^{-1} for the anion "carrier" of erythrocyte membranes, which might fulfill the structural requirements mentioned above. It is an open question of nomenclature how such translocators are to be classified. They resemble pores because the larger part of the membrane's diameter is passed by diffusion through a hydrophilic channel, and they resemble carriers because movement of a membrane component is essential for the transfer event. In the spirit of (*2*) we would not classify such structures, if they exist, as carriers. They may be viewed as pores in which the selectivity filter [B. Hille, *J. Gen. Physiol.* **58**, 599 (1971)] constitutes a peculiar energy barrier.
10. T. U. L. Biber and M. L. Sanders, *J. Gen. Physiol.* **61**, 529 (1973).
11. A. W. Cuthbert [*J. Physiol. (London)* **228**, 681 (1973)] computed, from amiloride binding data, a channel density of 400 μm^{-2} [lower limit at 2.5 mM $(Na)_o$], and from this calculated turnover rates for individual Na channels of 2000 sec^{-1} at 2.5 mM $(Na)_o$ and 8000 sec^{-1} at 115 mM $(Na)_o$ (upper limits). These turnovers are integrated over open and closed times of a channel and therefore cannot be directly compared with our results.
12. This work was supported by Deutsch Forschungegemeinschaft as project C1 within SFB 38, and by the Humboldt Foundation.

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Size Limit of Molecules Permeating the Junctional Membrane Channels

Abstract. *The permeability of the cell-to-cell membrane channels in salivary gland cell junction* (Chironomus thummi) *was probed with fluorescent-labeled amino acids and synthetic or natural peptides. Molecules up to 1200 daltons pass through the channels with velocities depending on molecular size. Molecules of 1900 daltons or greater do not pass. This passage failure seems to reflect the normal size limit for junctional channel permeation; the channels continue to be permeated by the molecules up to 1200 daltons when these are mixed with the nonpermeant molecules. From this size limit a channel diameter of 10 to 14 angstroms is estimated.*

Since the finding that fluorescein, a molecule of 330 daltons, passes through a cell junction of low electrical resistance (*1*), other fluorescent and colorant substances have been used to probe junctional permeability properties (*2*, *3*). However, the range of questions that could be studied has been restricted by the small number of useful probes available. We have now enlarged the repertoire of probes with the aim of determining the size limit of molecules permeating the junctional membrane channels (*4*).

We set out to construct fluorescent conjugates which incorporate some of the desirable features of the popular tracer fluorescein, such as water solubility, nontoxicity, low cytoplasmic binding, and high fluorescent yield. To obtain conjugates of well-defined structure, we sought, for the nonfluorescent backbone, not only a molecule of known structure but one with few reactive sites, preferably only one. Thus, the primary amine group of the synthetic and natural peptides listed in Table 1 was coupled with the fluorescent dyes fluorescein isothiocyanate (FITC), dansyl chloride (DANS), or lissamine rhodamine B (LRB) (*5*). The conjugation reactions were carried out at room temperature in an aqueous-acetone solvent made alkaline with $KHCO_3$. The products were purified by ion-exchange and gel-permeation chromatography. The criterion for purity was the formation of a single fluorescent spot in paper electrophoresis. Amino acid analysis and end-group analysis were performed on all peptides, except microperoxidase. The purified compounds were dissolved in water and the *p*H was adjusted to about 7 with KOH or HCl.

The solutions of the fluorescent probes were injected into cells of isolated *Chironomus* salivary glands (mid-fourth instar) with the aid of a micropipette and a pneumatic pressure system (*6*); the spread of the fluorescence inside the cells (excited with wavelengths of 460 nm for FITC, 340 nm for DANS, and 540 nm for LRB) was observed and photographed in a microscope darkfield, or, for velocity determinations, the spread was viewed and videotaped with the aid of an image intensifier-television system (*7*). The fluorescent emissions of FITC and DANS peak at 520 and 525 nm (yellow-green) and that of LRB, at 590 nm (red). Thus, in experiments where two tracers were injected together, the LRB was easily distinguished from either of the other two by the use of appropriate filters. In some cases, the tracer studies were combined with measurement of electrical coupling. Electrical current was then passed between the exterior and the interior of the cell injected with the tracer, and the resulting changes in membrane potential were measured in this cell and the immediate neighbor with the use of three microelectrodes (*1*).

The results obtained with the various tracers are summarized in Table 1. The amino acids and peptides with sizes less than or equal to 1158 daltons passed through the junction. Their fluorescence spread from the site of injection throughout the injected cell and into the cell neighbors at rates inversely related to molecular size. The tracers generally crossed several cell junctions on either side. The arrival of a tracer at the junction was marked by an abrupt change in the velocity of the fluorescence spread. With molecules less than or equal to 380 daltons, passage through the first junction became detectable within a few seconds of the tracer's arrival at the junction; and the fluorescence on the two sides of the junction appeared to equalize within 1 to 10 minutes. With molecules between 593 and 1158 daltons, the transit through junction was slower (tak-

ing up to 1 minute in some cases), and large differences in concentration across the junction persisted for at least 30 minutes, indicating a marked constraint on the transjunctional diffusion.

Among the junction-permeant molecules, FITC(Leu)$_3$(Glu)$_2$OH (1004 daltons) (Leu, leucyl; Glu, glutamyl) seemed to be a marginal case; it passed detectably through junction only in 2 out of 23 trials. Presumably this molecule is close enough to the upper size limit where small differences in configuration or charge become critical for channel permeation. The molecule, however, did not itself cause alteration in the channel permeability; LRB(Glu)$_3$OH (950 daltons) or LRB(Leu)$_3$(Glu)$_2$OH (1158 daltons), injected simultaneously with the FITC(Leu)$_3$(Glu)$_2$OH, traversed the junction, at the same time that the last-mentioned failed to do so.

The cell-to-cell passage of all tracer molecules was blocked when the cytoplasmic free Ca^{2+} concentration at the junction was elevated to about $10^{-4}M$ by injection of Ca^{2+} into the cells (together with the tracers), or by treating the cells with 5 m*M* cyanide, or perforating the cell membrane in Ca medium. In all these circumstances, the passage of the small inorganic ions is also blocked (*7*, *8*).

The peptides greater than or equal to 1926 daltons did not flow detectably through the junction (*9*). The junctions of the cells injected with these peptides, however, continued to be permeated by the smaller molecules. This was routinely checked by simultaneous or successive injection of a small molecule of a different fluorescent color. Thus, in the example illustrated in Fig. 1, the same junction was probed simultaneously with FITC-fibrinopeptideA (1926 daltons) and LRB(Leu)$_3$(Glu)$_2$OH (1158 daltons). The latter traverses the junction; the former does not.

The results suggest that, for peptides, the cutoff for permeation of the junctional membrane channels is about 1200 to 1900 daltons. An earlier estimate of the cutoff limit based on the junctional passage of FITC–serum albumin (*2*) is now shown to be wrong. Most likely the protein was degraded in the cells, and an FITC-labeled fragment (with the antigenicity of the original molecule) went through the junction.

To serve as a junctional tracer, the molecule must not be readily taken up by the cells from the exterior. All of the tracers listed in Table 1 met this condition, which was ascertained by immersing the glands for 0.5 to 1 hour in media containing the tracers at concentrations of $5 \times 10^{-4}M$ and then washing them. No fluorescence was visible in normal cells. In fact, these cells stood out as dark bodies in the fluorescent tracer media (*10*). This excluded extracellular pathways for the amounts of label seen transferred from cell to cell in the experiments. By contrast, when the glands were immersed, for example, in rhodamine B (not LRB) or DANS-cadaverine, the normal cells became fluorescent. These substances were rapidly concentrated in the cells and were therefore not useful as junctional probes.

In the case of the larger permeating molecules, the possibility needed to be considered that we were tracing a large labeled fragment (*11*) in which, say, one or two amino acids of the original molecules were missing because of enzymatic degradation within the cells. This possibility could be reasonably excluded by experiments in which LRB(Leu)$_3$(Glu)$_2$-OH, LRB(Gly)$_6$OH, and LRB(Glu)$_3$OH were respectively incubated for 2 to 12 hours with the cytoplasm of mashed cells (*12*). Portions were removed periodically from the incubating material and spotted for electrophoresis at *p*H 8.4 (0.05*M*

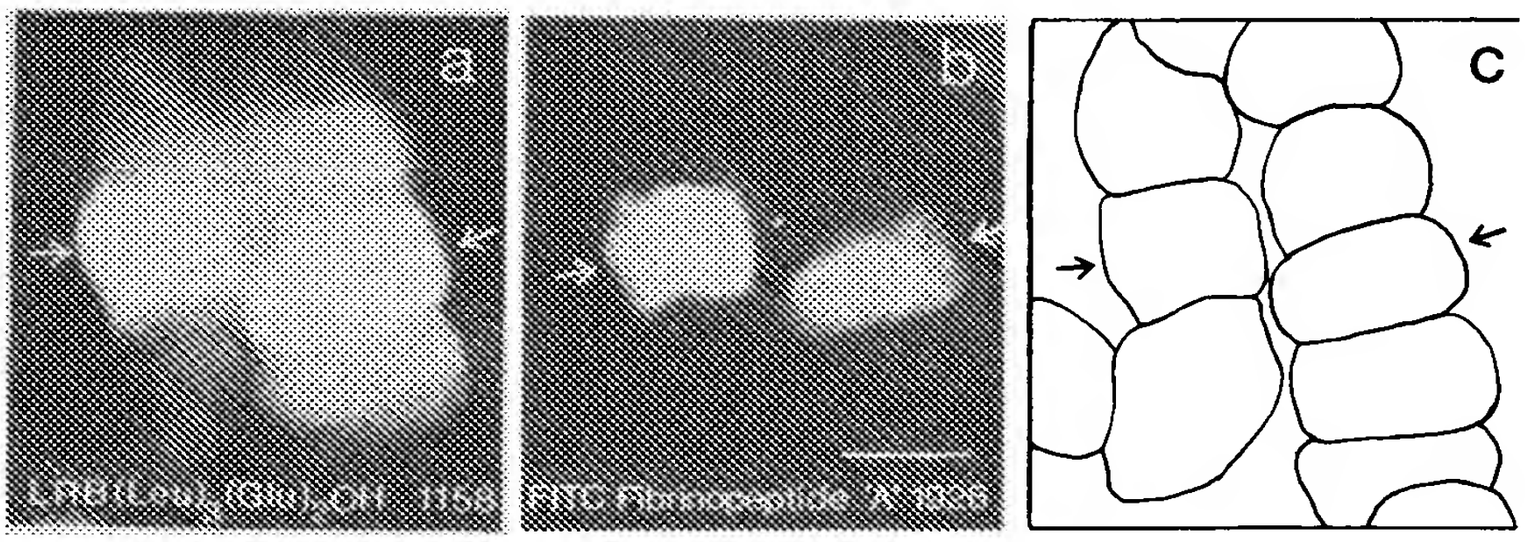

Fig. 1. Probing junctional membrane channels with two molecules close to the size limit of permeation. The red fluorescent tracer LRB(Leu)$_3$(Glu)$_2$OH (1158 daltons), the largest permeant molecule of the present series, is injected into the cells marked with an arrow, together with the yellow-green fluorescent tracer FITC–fibrinopeptide A (1926 daltons). (a) Distribution of the red tracer (1158 daltons) photographed (darkfield) in black and white 40 minutes after injection. (b) Distribution of the yellow-green tracer (1926 daltons) 45 minutes after injection. (The two fluorescences are set apart by the use of different excitation wavelengths and barrier filters.) The red tracer spread from the injected cells to several neighbors; the yellow-green tracer stayed within the injected cells (calibration bar, 100 μm).

Table 1. Cell-to-cell passage of fluorescent probes. Abbreviations: (Glu)OH, glutamic acid; (Gly)OH, glycine; (Leu)OH, leucine; (Tyr)OH, tyrosine.

Molecule	Size (daltons)	Cell-cell spread cases*	Control molecule†
DANS(SO_3H)	251	2 (2)	
DANS(Glu)OH	380	12 (12)	
LRB(SO_3H)	559	35 (35)	
DANS(Gly)$_6$OH	593	7 (7)	
DANS(Glu)$_3$OH	640	12 (12)	
LRB(Glu)OH	688	13 (13)	
FITC(Gly)$_6$OH	749	2 (2)	
FITC(Glu)$_3$OH	794	45 (45)	
DANS(Leu)$_3$(Glu)$_2$OH	849	12 (14)	
LRB(Gly)$_6$OH	901	13 (13)	
LRB(Glu)$_3$OH	950	13 (15)	
LRB(Glu-Tyr-Glu)OH	982	2 (2)	
FITC(Leu)$_3$(Glu)$_2$OH	1004	2 (23)‡	
LRB(Leu)$_3$(Glu)$_2$OH	1158	50 (56)	
FITC fibrinopeptide A	1926	0 (6)	LRB(Glu)OH LRB(Leu)$_3$(Glu)$_2$OH
FITC microperoxidase	2268	0 (1)	LRB(Glu)OH
FITC insulin A chain	2921	0 (10)	LRB(Glu)OH
DANS insulin A chain	3232	0 (3)	§
FITC insulin B chain	3897	0 (7)	LRB(Glu)$_3$OH
LRB insulin A chain	4158	0 (7)	Fluorescein (330 daltons)

*Parentheses give the number of trials. †Small molecule (spreading) used as control for same junction. ‡In seven cases of "no spread" of this tracer, LRB(Glu)$_3$OH or LRB(Leu)$_3$(Glu)$_2$OH was used as control molecule and found to spread through same junction. §Not tested with control molecule, but junction was coupled electrically.

NH_4HCO_3). The result was invariably a single fluorescent spot with the mobility of the original labeled peptide.

Our results thus provide a molecular weight limit for permeation of the junctional membrane channels. The permeating molecules were all short, simple, water-soluble peptide chains and hence were of extended form. Without further physical studies of the molecules themselves, it is not possible to determine from this weight limit a precise bore size of the channels. But the approximate bore size can be bracketed between the sizes of two limiting geometries of the largest permeant molecule, a sphere, representing the largest cross section, and a prolate spheroid with a major diameter of 30 Å, the upper limit of molecular extension. Thus, the effective channel diameter lies approximately between 14 and 10 Å (*13*). This is in satisfying agreement with a coarser estimate of the channel size based on electrical measurements. This estimate, based on the conductance of a minute junctional area (including the conductance component due to electrostatic interaction between channels) and the spacing of intramembranous particles of gap junction (widely assumed to contain the channels), gave a lower limit of conductance of 10^{-10} mho for the junctional channel unit and a lower limit of the channel diameter of the order of 10 Å (*14*).

IAN SIMPSON
BIRGIT ROSE
WERNER R. LOEWENSTEIN
Department of Physiology and Biophysics, University of Miami School of Medicine, Miami, Florida 33152

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4. For a definition of the junctional channel unit, see W. R. Loewenstein [*Ann. N.Y. Acad. Sci.* **137**, 441 (1966)] and (*14*)]; for its morphological aspects, see N. B. Gilula [in *The Nervous System*, D. Tower, Ed. (Raven, New York, 1975), vol. 1], D. Goodenough [*Cold Spring Harbor Symp. Quant. Biol.* **40**, 37 (1975)], C. Peracchia and M. E. Fernández-Jaimovich, [*J. Cell Biol.* **67**, 330a (1975)].
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6. The concentrations of the fluorescent compounds inside the micropipettes were $10^{-2}M$. The steady state concentrations inside the cells (upon dilution of the injected solutions into an average of ten cells) are estimated to range from $10^{-4}M$ to $10^{-5}M$.
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9. The pattern of spread of these molecules here in the normal, untreated cells was like that of the smaller molecules (≦ 1158 dalton) in cells whose cytoplasmic Ca^{2+} concentration was elevated—the tracers spread throughout the injected cell, but not beyond its junctional boundaries.
10. On the other hand, the gland lumen, which is open to the exterior through the gland duct and has a depth comparable to the cells, became strongly fluorescent. Furthermore, dead cells became as fluorescent as the duct under these conditions. Dead cells were recognized in bright field by their swollen and granular appearance, and their enlarged nucleus and chromosomes.
11. A small labeled fragment seemed unlikely because, as was already mentioned, the rates of intracellular movement of the tracer varied inversely as their presumed molecular weights.
12. In this condition one would expect maximum lysosome breakage and, hence, maximum peptidase activity.
13. The size ($2r$) for the spherical molecular shape was determined from

$$r = \left(\frac{3 \text{ mol wt} \times \bar{v}}{4\pi N} \right)^{1/3}$$

where N is Avogadro's number and $\bar{v}$ is the specific volume assumed to be 0.7; and the size for the most extended shape, was determined as the small diameter of the corresponding prolate spheroid whose major diameter was obtained with the aid of molecular models. The actual channel bore lies probably closer to the upper bracketing value (14 Å); for the molecules labeled with LBR and FITC, the small diameter of a realistic axiosymmetric equivalent is fixed at about this value by the labels themselves.
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15. We thank Dr. L. Lorand for suggestions and for fibrinopeptide A and Dr. S. J. Socolar for discussion. This work was supported by research grant CA 14464 from the Public Health Service.

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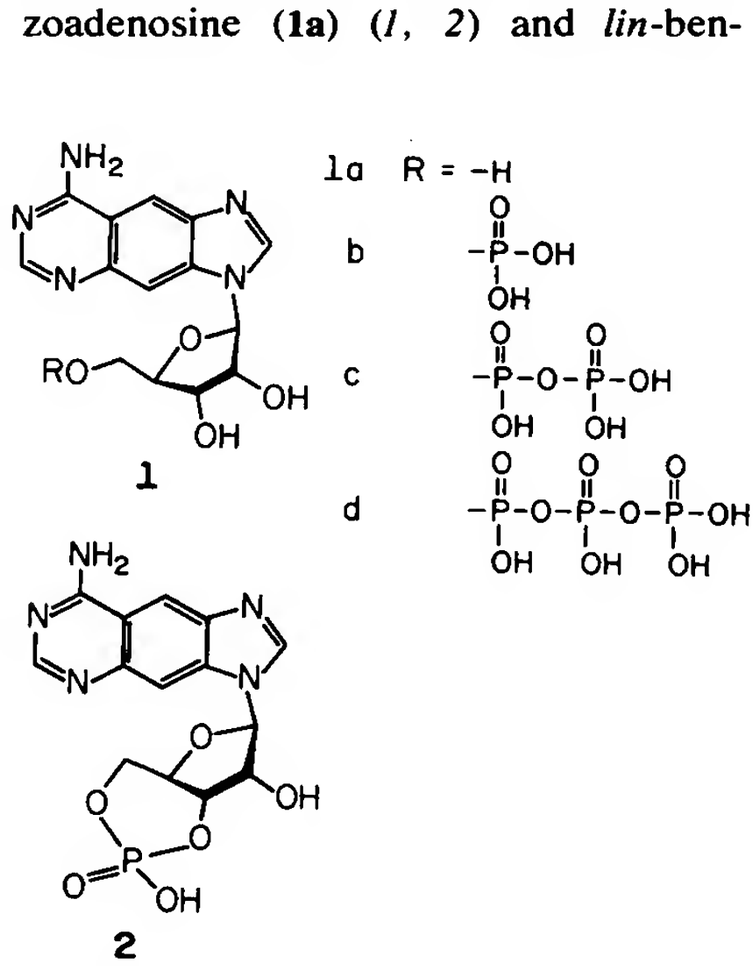
Defined Dimensional Changes in Enzyme Cofactors: Fluorescent "Stretched-Out" Analogs of Adenine Nucleotides

Abstract. *A concept is presented for testing the dimensional restrictions of enzyme-active sites by stretching the substrate or cofactor by known magnitude. These restrictions of enzyme-active sites specific for purine cofactors were tested by the synthesis and evaluation of* lin*-benzoadenosine 5'-triphosphate, 5'-diphosphate, and 3',5'-monophosphate with respect to enzyme binding and activity. These "stretched-out" (by 2.4 angstroms) versions of the adenine ribonucleotides bind strongly, slow the enzymatic rates, and have useful fluorescence properties.*

Laterally extended adenine nucleotides have been designed to examine the dimensional restrictions of enzyme-active sites specific for purine cofactors. One structural modification of this type involves the formal insertion of a benzene ring (actually four carbons) into the center of the purine ring system. In this way enzyme-binding characteristics at the terminal rings are preserved but are further separated by 2.4 Å while, at the same time, the potential for π interaction is increased. Initial experiments examining the substrate activity of *lin*-benzoadenosine (**1a**) (*1*, *2*) and *lin*-benzoadenine with a range of enzymes (*3*) demonstrated that such defined adjustments in the molecular periphery can help set limitations on the size and flexibility of the enzyme binding sites.

In view of these results it was anticipated that the enzymatic evaluation of the *lin*-benzoadenine nucleotides would also be informative since many enzymes utilize adenine nucleotides as substrates, cofactors, or allosteric effectors. In addition, it can be foreseen that the concept of defined dimensional changes is applicable to the construction and study of inhibitors. Furthermore, *lin*-benzoadenosine and its derivatives exhibit satisfactory fluorescence properties (a quantum yield of 0.44; a fluorescence lifetime of 3.7 nsec), and the nucleotides show sensitivity of the fluorophore to environmental conditions, such as divalent metal ions and stacking.



lin-Benzoadenosine (**1a**) was converted to its 5'-monophosphate derivative (**1b**) by reaction with pyrophosphoryl chloride according to the procedure described by Imai *et al.* (*4*). The integrity of the 5'-phosphorylation was established (i) by observing complete conversion of the *lin*-benzoadenosine 5'-monophosphate to the nucleoside (**1a**) on incubation with 5'-nucleotidase (*5*) and (ii) by ^{31}P NMR (nuclear magnetic resonance) spectroscopy.

lin-Benzoadenosine diphosphate (**1c**)